

# Epigenetic differences in cytogenetically normal versus abnormal acute myeloid leukemia

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Methylation of tumor suppression genes (TSGs) is common in myeloid malignancies. However, application of this as a molecular marker for risk stratification in patients with AML is limited. To elucidate the impact of patterns of TSG methylation on outcome in cytogenetically normal patients, 106 samples from patients with normal cytogenetic AML were evaluated for methylation of 12 genes by MSP. For sake of comparison, samples from patients with AML and abnormal cytogenetics (n = 63) were also evaluated. Methylation frequencies in the whole group (n = 169) were similar to previous reports for *CDH1* (31%), *ER* (31%), *FHIT* (9%), *p15<sup>INK4b</sup>* (44%), *p73* (25%) and *SOCS1* (75%). Methylation of *CTNNA1* was observed in 10%, *CEBP-α* in 16%, *CEBP-δ* in 2%, *MLH1* in 24%, *MGMT* in 11% and *DAPK* in 2% of AML samples. We find that DNA methylation was more prevalent in patients with normal compared to karyotypically abnormal AML for most genes; *CEBPα* (20% vs 9%), *CTNNA1* (14% vs 4%) and *ER* (41% vs 19%) ( $p < 0.05$  for all comparisons). In contrast, *p73* was more frequently methylated in patients with karyotypic abnormalities (17% vs 38%;  $p < 0.05$ ), perhaps due to specific silencing of the pro-apoptotic promoter shifting *p73* gene expression to the anti-apoptotic transcript. In AML patients with normal cytogenetics, TSG methylation was not associated with event free or overall survival in a multivariate analysis. In patients with AML, TSG methylation is more frequent in patients with normal karyotype than those with karyotypic abnormalities but does not confer independent prognostic information for patients with normal cytogenetics.

## Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by uncontrolled proliferation of primitive hematopoietic elements and recurrent cytogenetic abnormalities.<sup>1</sup> Specific genetic abnormalities (i.e., t(8; 21), inv(16), t(15; 17)) are pathognomonic and prognostically important in patients with AML. However, most patients with AML have a normal karyotype and, in these patients, it is often difficult to predict long-term outcome.<sup>2,3</sup> The prognostic role of novel molecular markers, including mutations of *FLT3* or *NPM1* genes and DNA methylation of *p15<sup>INK4b</sup>*, *ER* or *CDH1* have been reported in AML patients.<sup>4-6</sup> While the former have been adopted into widespread clinical practice, methylation of tumor suppressor genes (TSGs) has not yet proven useful as a prognostic marker, since previous studies have included heterogeneous patient populations and there has been a failure to control for the cytogenetic profile.<sup>7,8</sup>

Advances in chemotherapy and transplantation have substantially improved long-term survival in children with AML, but these advances have not similarly improved the outcomes for adults. Strategies to identify patients who are likely to relapse may be useful in developing new treatment algorithms.<sup>3</sup> This goal is particularly important in the large (~50%) fraction of adult AML patients presenting with normal cytogenetics, in whom

conventional treatment does not initially include bone marrow transplantation. Since these leukemias lack gross chromosomal (genetic) alterations, it is plausible that epigenetic changes may be more important in driving the pathogenesis in this setting. Previous work suggesting that methylation of TSGs may be prognostically important in patients with AML is also of interest in light of novel demethylating therapies (i.e., 5-azacytidine and 5-aza-2'-deoxycytidine) with documented efficacy in myeloid malignancies.<sup>9-12</sup> However, few, if any, studies have confirmed these previous reports, suggesting the possibility of positive publication bias, and none have limited the analysis to genetically homogenous patients.

This study was designed to assess methylation of genes with previously published biologic rationale and/or prognostic significance in AML. We chose this technique, rather than a genome wide strategy, due to the small number of patients in this study and the significant risk of false discovery inherent in these broader assessments. Our focus was to validate genes known to play a role in AML rather than to identify new genes for this unique set of patients. Methylation specific PCR (MSP) was used as the technique to assess gene specific methylation since this has been shown to detect methylation which is associated with alterations in expression of associated genes. (see **Suppl. Table 1** for references by gene).

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**Table 1.** Characteristics of the study population

Characteristics	All (n = 169)		Normal (n = 106)	
	Number	Percent	Number	Percent
<b>Cytogenetics</b>				
Good	10	6		
Normal	106	63	106	100
Intermediate	14	8		
Single Adverse	9	5		
Complex	30	18		
Age, (yrs) median and IQR	61 (48–70)		61 (51–70)	
White blood cell count (mean)	35,838		40,302	
<i>NPM1</i> mutation	26	15	23	22
<i>FLT3-ITD</i> mutation	47	28	32	30

In order to examine the role of DNA methylation of TSGs in providing prognostic information we (a) determined the frequency of methylation of 12 TSGs across cytogenetic and molecular (*FLT3* and *NPM1* mutant) risk groups, (b) compared the methylation frequencies for AML patients with normal karyotype de novo versus those with antecedent cytopenias/myelodysplastic syndrome (MDS), (c) compared methylation frequencies for patients with normal karyotype AML at the time of diagnosis to those presenting with normal karyotype AML at the time of relapse and (d) evaluated the impact of methylation of TSGs as a prognostic biomarker of clinical outcome in uniformly treated (cytarabine-based chemotherapy) AML patients with a normal cytogenetic profile. **Supplementary Table 1** details the chromosomal location of the 12 genes selected, their previously published rates of methylation and the rationale for their importance in AML.

## Results

**Patient characteristics.** A total of 169 AML patient samples were collected, of which 106 had normal cytogenetics. **Table 1** details demographic information for the patients. Median age was 61 for both groups. Of the normal cytogenetic samples, 34 were procured at relapse and 72 were procured from patients at initial diagnosis. These 72 samples (from patients at initial AML diagnosis) were annotated for treatment received. All 72 patients received cytarabine based induction therapy, with the majority of patients receiving combination therapy with cytarabine and daunorubicin. Of the 72 patients, there were 39 with de novo AML (four received FLAM,<sup>17</sup> three received the Linker regimen,<sup>18</sup> six received standard “7 plus 3” and 26 received ACDVP16<sup>19,20</sup>) and 33 patients had antecedent MDS (five received FLAM and 28 received ACDVP16). No patients received epigenetic therapy with 5-azacytidine or deoxy-azacytidine prior to sample analysis. Since all 72 patients were administered a cytarabine based induction regimen, their event free and overall survival was assessed and outcomes were correlated with methylation of individual genes or numbers of genes. These results were analyzed in a

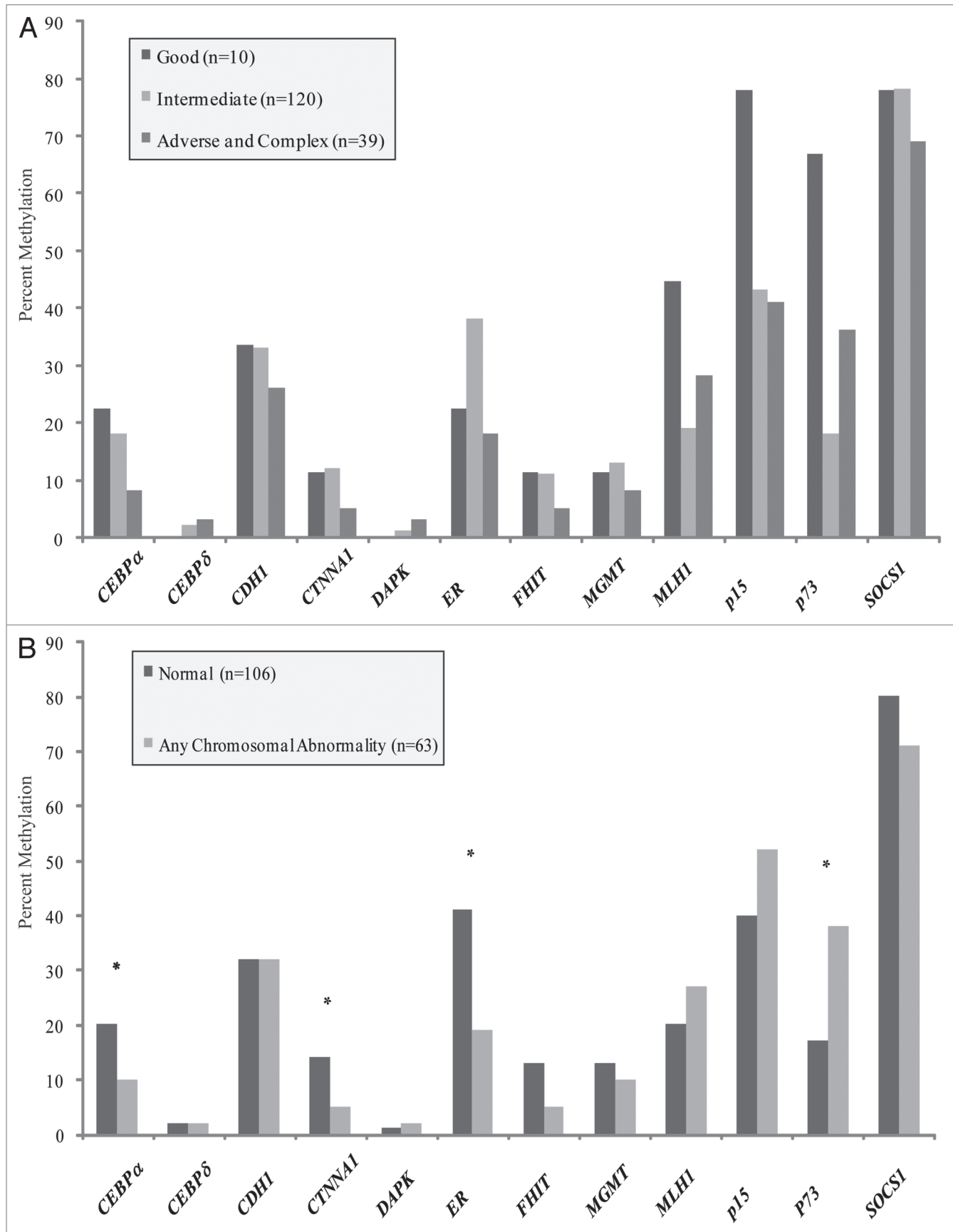
multivariate model including age, total WBC count at diagnosis (greater or less than 50,000/uL as a dichotomous variable), antecedent cytopenias, *NPM1* mutations and *FLT3-ITD* status. Age was assessed as a continuous variable. (**Table 1**)

**Methylation frequency by gene.** The observed methylation frequencies for our AML samples was similar to that in previous studies (see **Suppl. Table 1**) for *CDH1* (31%; 53/169), *ER* (31%; 52/169), *FHIT* (9%; 16/169), *p15<sup>INK4b</sup>* (44%; 74/169), *p73* (25%; 42/169) and *SOCS1* (75%; 127/169). Methylation of *CTNNA1* was observed in 10% (17/169), *CEBP-α* in 16% (27/169), *MLH1* in 24% (41/169) and *MGMT* in 11% (19/169) of AML samples; these genes have rarely been reported in the literature previously. Methylation of *CEBP-δ* (2%; 3/169) and *DAPK* (2%; 2/109) was rare. **Figure 1A** displays methylation frequencies by karyotypic risk group. *SOCS1* and *p15* were the most frequently methylated genes, followed by *p73* and *CDH1*. No significant differences were seen in methylation frequency between different (low, medium, high) cytogenetic risk groups.

**Comparisons between methylation patterns by epigenetic and genetic alterations.** While the previous analysis had suggested no distinct differences between varying cytogenetics risk groups, we sought to determine whether the overall level of genetic alterations were associated with any differences in the frequency DNA methylation. To do so, we compared all normal karyotype patients to those with any karyotypic abnormality. **Figure 1B** demonstrates methylation frequencies for the patients with normal cytogenetics (n = 106) and those with any karyotypic abnormality (n = 63), consisting predominantly of patients with complex karyotype (n = 30). TSG methylation was more frequent (p < 0.05) in patients with normal cytogenetics than those with a karyotypic abnormality for *CEBP-α*, (20% vs. 9%), *CTNNA1* (13% vs. 4%) and *ER* (41% vs 19%) (**Fig. 1B**). In contrast, only *p73* methylation was more common in AML samples with a cytogenetic abnormality (38% versus 17% in normal karyotype; p < 0.05). Methylation of this gene was particularly common in samples with a complex karyotype, with 41% of these leukemias having *p73* methylation. It is also noted that frequency of methylation of *p73* was 70% in the good risk cytogenetic group, of which five were APL from the total of ten samples examined.

**Methylation frequency in *FLT3* and *NPM1* mutated AML.** We next examined whether the differences in DNA methylation observed for gross genetic changes, that is cytological rearrangements, would also be observed with smaller genetic alterations. The two most common mutational changes in AML involve *FLT3* and *NPM1*. **Figures 2A and B** demonstrate the relative frequency of methylation by gene for patients with and without these abnormalities. No significant differences were found in the frequency of any TSG methylation according to the presence of *FLT3-ITD* or *NPM1* mutations, suggesting that the altered DNA methylation patterns observed with chromosomal rearrangements did not also include these individual gene mutations.

**Methylation frequency by antecedent hematological diagnosis in normal karyotype AML.** In patients with AML, the presence of an antecedent hematologic condition (i.e., MDS, myeloproliferative disorder or cytopenias >6 months prior to diagnosis) portends a worse prognosis.<sup>21</sup> Furthermore, such an



**Figure1.** For figure legend, see page 593.

**Figure 1 (See opposite page).** (A) Methylation rates by gene for each of the genes examined across the recognized cytogenetic risk groups. The good risk group includes 10 patients with t(8; 21), inv(16) or t(15;17) mutations, the intermediate group includes 106 patients with normal karyotype leukemia and 14 patients with <3 karyotypic abnormalities, the adverse group includes 30 patients with complex karyotype and 9 patients with abnormalities involving chromosomes 5, 7 or 11q23. No significant differences were observed between each of these cytogenetic risk groups. (B) When patients with normal karyotype were compared to those with a structural karyotype abnormality, differences in methylation frequency were observed. Methylation of *CEBP-α*, *CTNNA1*, *ER* and *p73* (\*) were significantly different between the two groups ( $p < 0.05$ ).

antecedent diagnosis has been associated with an increased rate of TSG methylation.<sup>11</sup> Within our group of patients uniformly treated with high-dose chemotherapy, no significant differences were observed between the rates of TSG methylation in normal karyotype AML patients with ( $n = 33$ ) and without ( $n = 39$ ) a history of antecedent cytopenias/MDS (Fig. 3A).

**Methylation frequency in normal karyotype aml: diagnostic versus relapsed samples.** No significant differences in methylation were observed between normal karyotype samples obtained from patients at the time of diagnosis ( $n = 72$ ) and those obtained from patients at the time of AML relapse ( $n = 34$ ). A trend towards increased methylation of *p73* was appreciated in the relapsed samples (13% vs. 26%) (Fig. 3B).

**Implication of *p73* methylation frequency in samples with cytogenetic abnormalities.** Of the 12 genes examined, only *p73* was more frequently methylated in samples with an abnormal rather than a normal karyotype, and this difference was statistically significant ( $p < 0.04$ ). The abnormal karyotype was frequently noted to be associated with the complex cytogenetic profile rather than a monosomy alteration. One explanation for this is the complex transcripts generated from the *p73* gene, depicted in Figure 4A demonstrating the two different transcripts, highlighting the promoter regions for each and the location of the CpG island. The long transcript, originating from the 5' promoter, TAp73, is transcribed from a promoter within a CpG island.<sup>16</sup> DNA methylation of this promoter results in silencing the pro-apoptotic TAp73 gene transcript, while potentially allowing retained expression of the anti-apoptotic DNp73 transcript. Previous studies only demonstrated loss of the longer or full-length transcript<sup>16</sup> since dual promoter usage was not known at the time of identification of silencing of *p73*. To test this, we examined several cell lines to determine if methylation of *p73* results in loss of the TAp73 transcript with preserved DNp73 expression.

KG1a and U937 leukemia cell lines have complete promoter region methylation of *p73*, and silencing of the TAp73 gene transcript (Figs. 4B and C). In KG1a there was continued expression of the DNp73 gene transcript, while in U937 this transcript was also silenced. The HL60 leukemia cell line demonstrated hemimethylation of *p73* with a corresponding decrease in the TAp73 gene product, but with significant expression of the DNp73 gene product. ML1 was entirely unmethylated and had normal levels of both TAp73 and DNp73. Treatment of KG1a and U937 cell lines with 1  $\mu$ M 5-azacytidine for 72 h resulted in reversal of methylation of the *p73* gene (Fig. 4D) and re-expression of the TAp73 transcript (Fig. 5E). Treatment with 300 nM suberoylanilide hydroxamic acid (SAHA) did not reverse methylation nor did this result in re-expression of the TAp73 gene product.

**TSG methylation as a prognostic marker.** Methylation of the 12 TSGs analyzed did not correlate with outcome in our

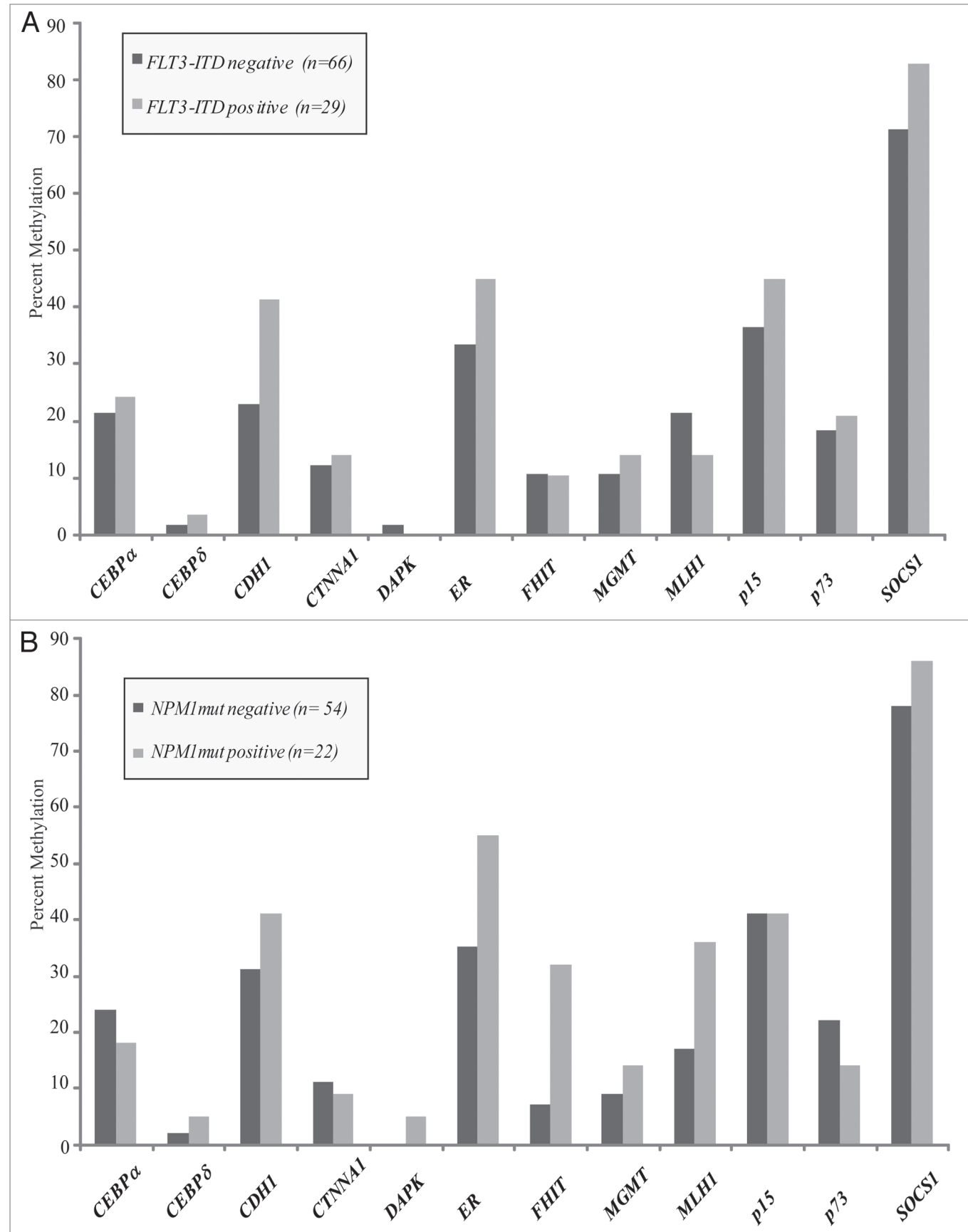
uniformly treated, newly diagnosed patients with normal karyotype. Figure 5A and B demonstrate multivariate hazard ratios for relapse and death by gene in this group. Methylation of *p73* and *MLH1* demonstrate a trend towards an adverse impact upon disease free survival, however this finding was not statistically significant. We examined the impact of methylation of multiple TSGs in concert and found no statistically significant impact of methylation of multiple genes on outcome in this sample of uniformly treated patients. Furthermore, there was no correlation between primary refractory disease status after high-dose induction chemotherapy and methylation of individual TSG or groups of TSGs (data not shown).

## Discussion

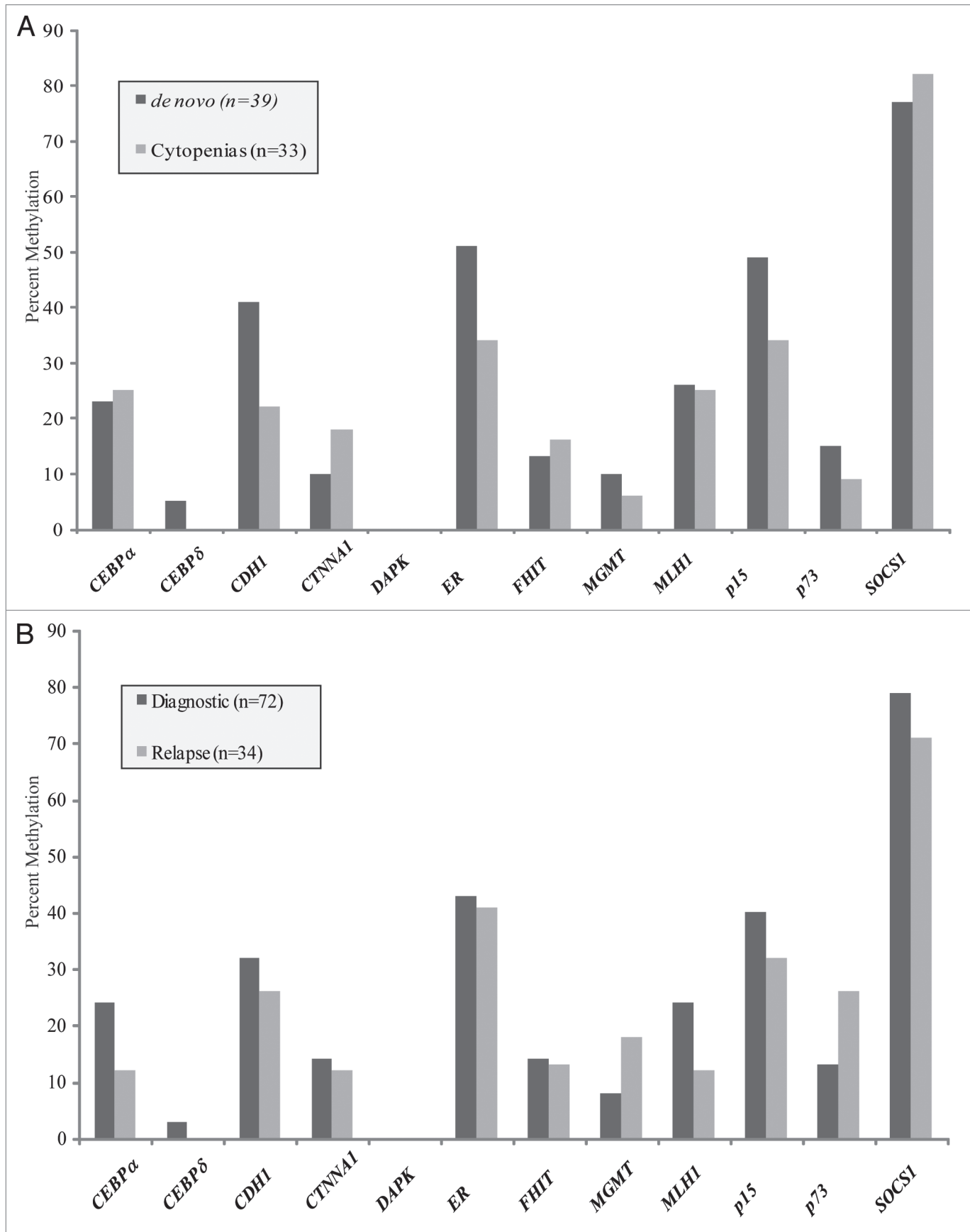
Over 50% of patients diagnosed with AML have normal cytogenetics. In these patients, current prognostic indicators do not predict those patients at higher risk of relapse who would benefit from initial therapy with an experimental regimen or a bone marrow transplant. Methylation of TSGs has been reported to predict poor outcome in patients with AML.<sup>5</sup> Our data suggest that methylation of TSGs is more frequent in AML patients with normal karyotype than those with karyotypic abnormalities; however, we found no evidence that methylation of any one of the 12 genes (or group of genes) correlated with outcome in AML patients with normal cytogenetics that were uniformly treated with standard cytarabine based induction chemotherapy. Our findings may differ from previous reports because we studied a more uniform patient population. Specifically, previous reports have been limited by sample selection and heterogeneity of both disease and treatment.<sup>5,7,12</sup> Our study carefully details the methylation of potentially important TSGs in a uniformly treated population of patients within the recognized paradigm of cytogenetic and molecular risk groups in AML.

Comparison of normal karyotype patients to those with structural abnormalities was performed with the rationale that structural/genomic chromosomal changes may “trump” the biological impact of epigenetic gene silencing. Methylation frequency of *CEBP-α*, *CTNNA1*, *ER* and *p73* differed ( $p < 0.05$ ) between patients with normal cytogenetics and those with karyotypic abnormalities. For all genes except *p73*, TSG methylation was more common in patients with a normal karyotype than in those with any cytogenetic abnormality (Fig. 1B). These differences suggest that patients with a normal karyotype (e.g., lacking large genetic changes) may have a greater degree of epigenetic alteration(s).

Although there are reports documenting the acquisition of promoter hypermethylation at the time of relapse for individuals sampled serially we found no difference in the rates of



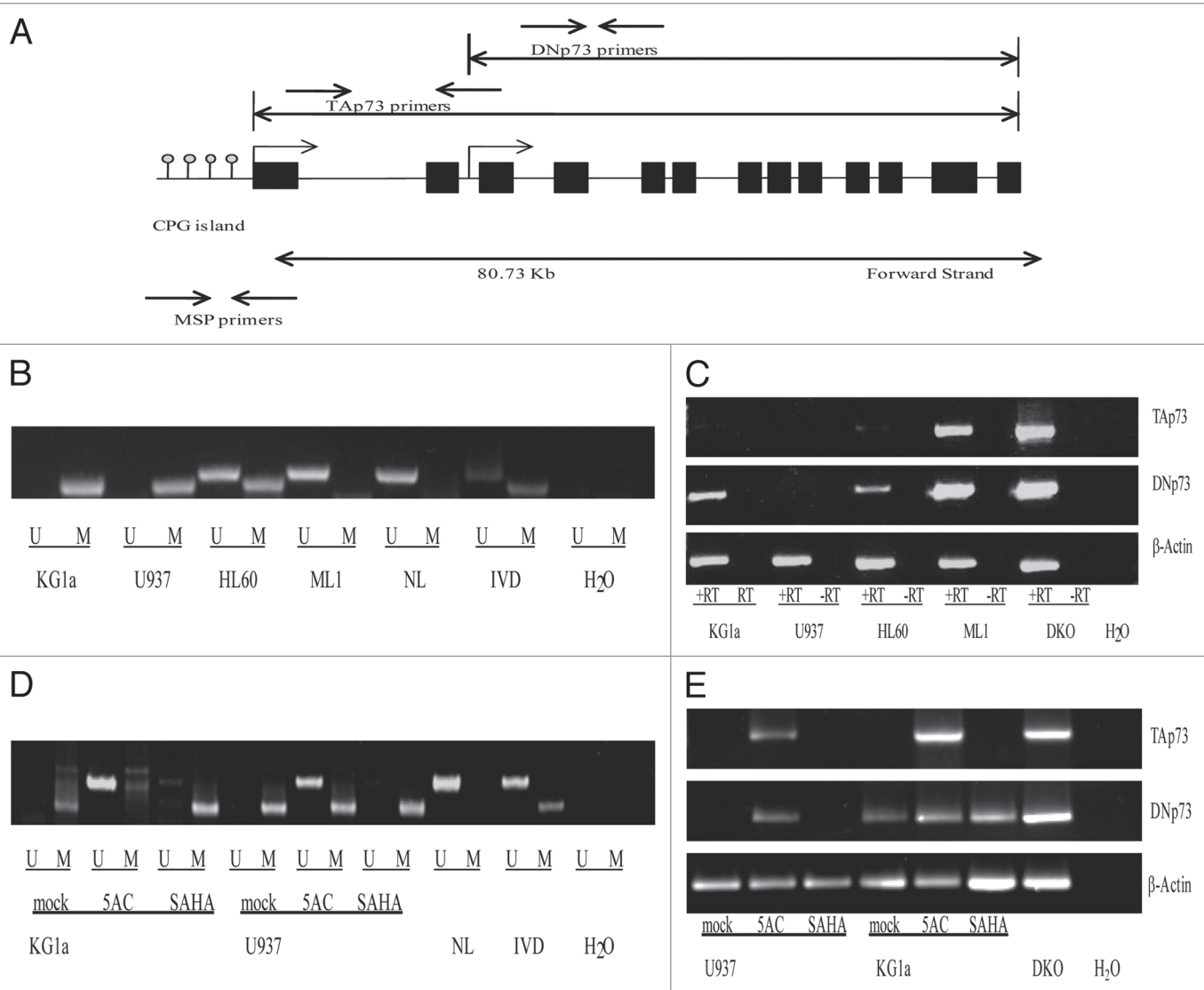
**Figure 2.** (A) No significant differences were observed between rates of methylation for normal karyotype samples with and without a *FLT3-ITD* mutation. (B) No significant differences in rates of methylation were observed between samples with and without a *NPM1* mutation.



**Figure 3.** For figure legend, see page 596.



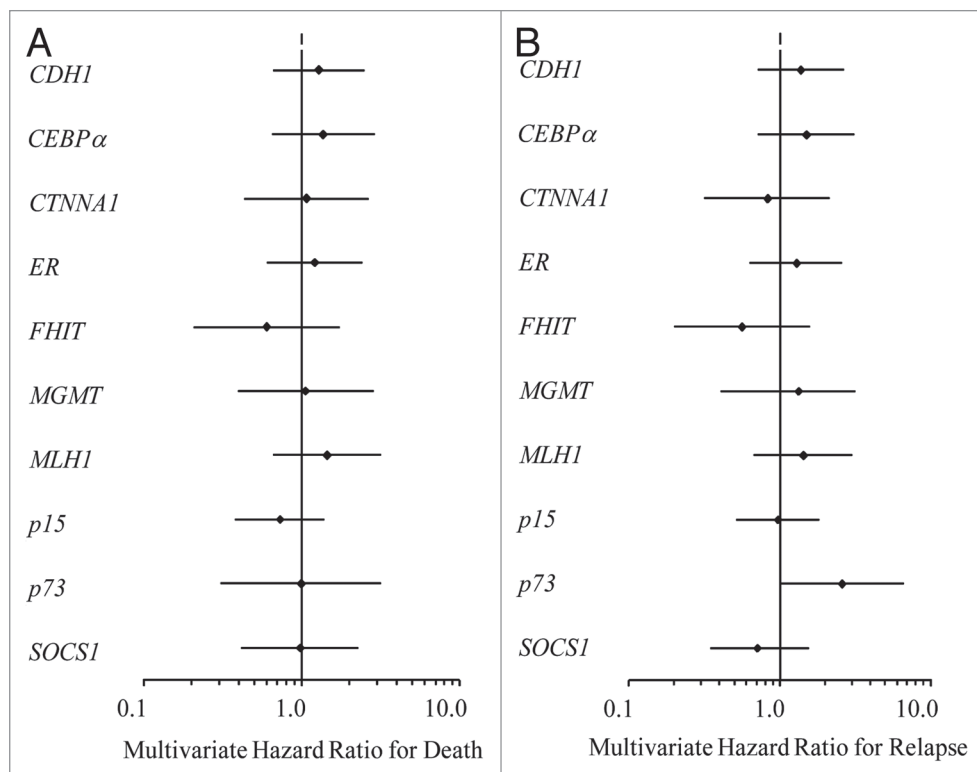
**Figure 3 (See previous page).** (A) No significant differences were seen between samples obtained from patients with de novo leukemia and those presenting with a history of an antecedent hematologic abnormality. Patients in the latter group were older, with a median age of 69 years vs. 52 years in the de novo group, and all had a diagnosis of AML at the time of sample acquisition. (B) There were no differences in methylation between normal karyotype samples obtained at the time of diagnosis as compared to those obtained at the time of AML relapse.



**Figure 4.** (A) Schematic of *p73* gene demonstrating its 14 exons and the location of our MSP and RT-PCR primers. The TAp73 transcript includes exons 1–14 and has a dense CpG island upstream of the transcription start site. The DNp73 transcript includes exons 3–14. (B) Methylation Specific PCR reactions for leukemia cell lines KG1a, U937, HL60 and ML1. KG1a and U937 demonstrate complete methylation, HL60 is hemimethylated for *p73*, ML1 is unmethylated. NL is a negative control for methylation in normal peripheral blood lymphocytes and IVD is a positive control for methylation. (C) RT-PCR for the long (TAp73) and short (Dp73) transcripts of *p73*. TAp73 exerts a pro-apoptotic and DNp73 an anti-apoptotic effect. Methylation of the *p73* promoter preferentially silences the long transcript of *p73* while allowing continued expression of the short transcript, favoring an anti-apoptotic phenotype. (D) Treatment for 72 h with 1  $\mu$ M 5-azacytidine (5AC) reverses methylation, while untreated (mock) and SAHA treated cell lines maintain a methylated phenotype. (E) Treatment of KG1a and U937 cell lines with 5AC results in re-expression of silenced TAp73 (both) and DNp73 (KG1a).

methylation between normal karyotype samples from the time of AML diagnosis versus those obtained from patients at the time of relapse.<sup>22</sup> Since we evaluated two different patient populations (one group at diagnosis and one at relapse) this may explain the difference in our results from the previous literature. The heterogeneity between individual patients may be greater than the differences in methylation frequency that occur for individual

genes from the time of diagnosis to relapse. Furthermore, it may suggest that it is important to evaluate the change in methylation status (i.e., unmethylated to methylated) rather than frequency of methylation for each gene. Methylation of TSG predicts poor outcome in patients with AML.<sup>23</sup> We examined the impact of methylation of these genes on survival in the entire group and, in contrast to prior reports



**Figure 5.** Uniformly treated patients with normal karyotype AML at initial diagnosis (n = 72) (A) Multivariate hazard ratio for relapse by gene with 95% confidence intervals designated by black bars. (B) Multivariate hazard ratio for death by gene corrected for age, antecedent cytopenia(s) at diagnosis, *FLT3-ITD* and *NPM1* mutational status and total white blood cell count at time of AML diagnosis with 95% confidence intervals designated by black bars.

from other groups, found no evidence that methylation of any one gene correlated with outcome.<sup>7</sup> Previous reports in the literature are limited by sample selection and heterogeneity of both disease and treatment.<sup>5,7,12</sup> We therefore examined a significant number of AML patients with normal cytogenetics, all of whom had undergone standard induction chemotherapy at a single institution. Even within this more uniform population, methylation of the selected genes did not confer a statistically significant impact upon event free or overall survival.

Among the 12 genes examined, only *p73* was more frequently methylated in AML samples with an abnormal karyotype. Although this finding does not directly implicate methylation of *p73* in the development of chromosomal abnormalities, the association is particularly interesting since two different isoforms of *p73* (TAp73 and DNp73) appear to affect the resulting phenotype. The long transcript (TAp73) includes exons 1 and 2 of the gene and is transcribed from an upstream promoter possessing a typical CpG island, while the short transcript (DNp73) is transcribed from a distinct promoter near exon 3. These two isoforms appear to have pro and anti-apoptotic effects respectively, and loss of the long isoform, TAp73 results in a high incidence of spontaneous tumors and genomic instability in TAp73 deficient mice.<sup>24</sup> This is consistent with the critical role of p73 in caspase-independent mitotic death,<sup>25</sup> suggesting that with loss of pro-apoptotic TAp73, cells would survive abnormal mitosis and accumulate

genomic alterations. The phenotype observed in KG1a and HL60 cell lines, loss of the TAp73 transcript and preservation of DNp73 transcription, may produce an environment permissive for genomic instability and provides an explanation for our observation that samples with cytogenetic abnormalities are more likely to have methylation of *p73*. While *p73* methylation is a relatively uncommon event in all patients with AML, the frequency of 41% of patients with a complex karyotype suggests that this may contribute to the acquisition of additional genomic abnormalities.

Our study has certain limitations. Although we did not identify epigenetic changes that predicted outcome in AML patients with normal cytogenetics, it is possible that methylation of genes not yet identified may be associated with adverse outcome in these patients. Improved technologies for identifying genetic and epigenetic alterations (HELLP assay and microarray studies) may help to identify markers that can be used for risk stratification of this population in the

future.<sup>26</sup> Of published studies, ours is one of the most comprehensive analysis of TSG epigenetic changes by MSP within the recognized paradigm of cytogenetic and molecular risk groups in AML. Nevertheless, it is limited by the small size of our patient population.

Although none of the genes examined in our study were associated with a statistically significant impact on prognosis, there was a trend towards adverse outcome for patients with *p73* and *MLH1* methylation, perhaps suggesting that functional loss of these genes may provide leukemia cells with the ability to tolerate DNA damage and thus confer chemotherapy resistance. Further investigation into the impact of *p73* methylation on genetic stability is necessary to verify this hypothesis.

## Design and Methods

**Cell lines.** Cell lines (DKO, HL60, KG1a, ML1 and U937) were purchased from American Type Culture Collection and grown in recommended media (ATCC, Manassas, VA, USA). Pelleted cells from each cell line were used for RNA and DNA extraction. DNA was extracted and bisulfite treated for analysis as described below.

**Patients, diagnosis and treatment.** One hundred and sixty nine freshly frozen bone marrow or peripheral blood samples collected between February 1998 and January 2008 from patients



with AML were obtained from the Johns Hopkins Leukemia bank. The diagnosis of AML was made by standard criteria<sup>13</sup> in 91 men and 78 women with a median age of 61 years (Table 1). All clinical samples and clinical data were obtained in accordance with HIPAA regulations as part of a protocol approved by the Institutional Review Board at the Johns Hopkins Hospital, and all patients signed informed consent according to Health and Human Services guidelines and the Declaration of Helsinki. Samples were annotated for patient age and white blood cell count (WBC) at diagnosis, cytogenetics by karyotype and FISH analysis, induction therapy and overall and event free survival. Clinical data for overall and event free survival was obtained for the 72 treated patients with normal cytogenetics and outcomes were correlated with methylation status for the genes in our panel.

**DNA extraction.** Freshly frozen bone marrow or peripheral blood samples were mixed with an equal volume of lysis buffer (50 mM Tris, 50 mM EDTA, 2% sodium dodecyl sulfate, 10 mg/mL protein kinase) and incubated overnight at 55°C. One half volume of lysis buffer was added daily until the sample had been completely digested. Once digestion was complete, DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), ethanol precipitated and re-suspended in 50–500 µL of double distilled water (ddH<sub>2</sub>O). DNA was quantified using the NanoDrop® ND-1,000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

**Bisulfite treatment.** Bisulfite modification of 1–2 µg of DNA per sample was performed using the Zymo Research EZ DNA methylation kit<sup>TM</sup> (Orange, CA, USA) as per manufacturer's instructions. Bisulfite treated DNA was re-suspended in 40–80 µL of ddH<sub>2</sub>O.

**Methylation-specific polymerase chain reaction.** Methylation-specific PCR primers for *CTNNA1*, *CEBP-α*, *CEBP-δ*, *DAPK*, *CDH1*, *ER*, *FHIT*, *MLH1*, *MGMT*, *p15<sup>INK4b</sup>*, *p73* and *SOCS1* have been published previously and were purchased from Integrated DNA Technologies (Coralville, IA, USA). All primers appear in **Supplementary Table 2**. MSP was carried out in 25 µL reaction volumes containing 10x MSP buffer (16.6 mM ammonium sulfate/ 67 mM Tris, pH8.8/6.7 mM MgCl<sub>2</sub>/ 10 nM 2-mercaptoethanol), 10 mM deoxynucleoside triphosphates, 0.5 µL of a 5 mM solution of each of the methylated or unmethylated primers, 0.5 µL of JumpStart<sup>TM</sup> REDtaq<sup>®</sup> DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) and 2–4 µL of bisulfite treated DNA.<sup>14</sup> DNA was amplified with the following protocol: 95°C for 5 min, followed by 35 cycles of 95°C for 30s, 58 or 60°C for 30s (58°C for all genes except *MGMT* and *MLH1* for which an annealing temperature of 60°C was utilized), 72°C for 30s and a final extension step of 72°C for 5 minutes. In vitro methylated DNA (IVD) was created by treating human cell line DNA with SSI-1 Methylase (New England BioLabs, Ipswich, MA, USA) as directed and used as a positive control following bisulfite treatment as described above. Normal lymphocytes collected from six healthy donors were used as a negative control. Seven and a half microliters of each amplification product were resolved on 2% agarose gel containing GelStar<sup>®</sup> Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA) and visualized under ultraviolet light (UV). Genes with a methylation frequency less

than 5% were excluded from our survival analysis due to a lack of statistical power.

**FLT3-ITD mutational analysis.** For qualitative analysis of *FLT3*-ITD mutations, genomic DNA was isolated as described above. PCR was performed using QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), with primers flanking the juxtamembrane coding region as previously published (Sense 5'-GCA ATT TAG GTA TGA AAG CCA GC-3', anti-sense 5'-CTT TCA GCA TTT TGA CGG CAA CC-3').<sup>15</sup> The following protocol was used: 95°C for 5 min, followed by 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min, and a final extension cycle of 72°C for 10 min. 10 µL of each amplification product was resolved on 2.5% agarose gel containing GelStar<sup>®</sup> Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA) and visualized under UV.

**NPM1 mutational analysis.** DNA corresponding to exon 12 on *NPM1* was amplified by PCR using the following primers: NPMF 5'-TTA ACT CTC TGG TGG TAG AAT GAA-3', NPMR 5'-CAA GAC TAT TTG CCA TTC CTA AC-3'. 25 µL reactions were prepared for each sample using 12.5 µL of QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), 7.5 µL of ddH<sub>2</sub>O, 0.5 µL of 5 mM solution of each primer and 4 µL (50–100 ng) of genomic DNA. Samples were amplified using the following protocol: 94°C for 10 min, 35 cycles of 94°C for 30s, 58°C for 30s, 72°C for 1 min and a final extension cycle of 72°C for 10 min. PCR amplification products were mixed with an equal volume of 2x formamide loading buffer (90% [v:v] deionized formamide, 1X sodium borate, 0.4% [w:v] each of xylene cyanol and bromophenol blue), incubated at 100°C for 3 min and loaded onto a 4.5% nondenaturing polyacrylamide sodium borate gel. Electrophoresis was performed at 100V for 4–6 h at 4°C. Gels were stained with ethidium bromide and PCR products visualized under UV.

**RNA extraction and RT-PCR.** RNA was extracted from cell lines using the RNeasy Mini Kit from Qiagen (Valencia, CA, USA) according to manufacturer's instructions. cDNA was made from RNA using Oligo dT<sub>(20)</sub> and the Superscript<sup>TM</sup> III RT enzyme (Invitrogen, Carlsbad, CA, USA). RT reactions were carried out with and without the RT enzyme to exclude the possibility of non-coding occult DNA sequences within the extracted samples. RT-PCR primers for p73 (TAp73) have been published previously<sup>16</sup>. Primers for the internal short transcript of p73 (DNp73) were designed and validated by the authors. Primers for TAp73 were 5'-CGG GAC GGA CGC CGA TG-3' (sense, exon 1) and 5'-GAA GGT CGA AGT AGG TGC TGT CTG G-3' (antisense, exon 3). Those for DNp73 were 5'-ACT AGC TGC GGA GCC TCT C-3' (sense, exon 3) and 5'-TGC TAG CAG ATT GAA CTG G-3' (antisense, exon 5). β-Actin was used as a positive control for gene expression. PCR reactions were optimized and carried out in 25 µL reaction volumes using 12.5 µL of QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), 7.5 µL of ddH<sub>2</sub>O, 0.5 µL of 5 mM solution of each primer and 4 µL (50–100 ng) of genomic DNA. Reaction conditions were 95°C for 15 min, 25 to 40 cycles of 95°C for 30 s (35 cycles for TAp73, 40 cycles for DAp73 and 25 cycles for β-Actin), 60°C for 30s, 72°C for 30s, and a final extension cycle of 72°C for 10 min. 8 µL of each amplification product were

resolved on 2.5% agarose gel containing GelStar® Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA) and visualized under UV.

**Statistical analysis.** All patient samples were obtained from the Johns Hopkins Leukemia Tumor bank. Clinical information was collected from the electronic patient record. Associations between individual gene markers and variables such as *NPM1* mutation, *FLT3* mutation, antecedent disease status and white blood cell count at diagnosis were assessed by means of logistic regression analysis. Two-sided p values <0.05 were considered to indicate statistical significance. Univariate and multivariable Cox proportional hazards regression models were utilized to assess the independent effect of DNA methylation on disease recurrence and mortality. Results of all regression models are reported as hazard ratios with corresponding 95% confidence intervals. To control for multiple testing, Bonferroni adjustment was used to correct for family-wise error rate. All statistical calculations were performed with the use of STATA 10 statistical package (College Station, TX, USA).

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## Conflict of Interest

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## Note

Supplementary materials can be found at:

[www.landesbioscience.com/supplement/GriffithsEPI5-7-sup.pdf](http://www.landesbioscience.com/supplement/GriffithsEPI5-7-sup.pdf)

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